REMARKS

By the present Amendment, independent claim 19 has been amended to recite a method of treating skin comprising applying a cosmetic composition consisting essentially of citrulline in an amount of 0.5 mM to 60 mM to skin so that active oxygen in the skin is scavenged by the citrulline. Dependent claim 20 has been amended to recite that the cosmetic composition consists essentially of citrulline in an amount of from 4 mM to 60 mM. Corresponding revisions have been made to independent claims 25 and 26 and new dependent claims 28 and 29 have been added to recite the range of citrulline of from 4 mM to 60 mM.

Support for the new claim language is provided in the specification. In particular, the ability of citrulline to scavenge oxygen is set forth throughout the specification and the quantitative evaluation of the scavenging effect of citrulline is described in the passage beginning with paragraph [0033]. Paragraph [0035] refers to Figure 4 and describes concentrations of various materials that inhibit 50 percent of hydroxylation of salicylic acid. From the results being shown in Table 1 of paragraph [0036], it will be understood that the 50 percent inhibition concentration for citrulline is 4.34×10^{-3} molar or 4.3 mM. Figure 4 illustrates concentrations up to 0.06 mM or 60 mM.

From the data provided in the specification and in Figure 4, it is clear to those of ordinary skill in the art that amounts below 4 mM are effective. To further demonstrate this point, the Examiner's attention is respectfully directed to the article entitled "The Application of ESR Spin-Trapping Technique to the Evaluation of SOD-

like Activity of Biological Substances".[1] While this article does not relate to citrulline, it includes a discussion of the kinetic competition with a 50 percent inhibitory dose (ID_{50}) involving the second-order rate constant for the reaction between an oxygen radical and a biological substance. In this regard, the Examiner's attention is directed to formula (9) on page 190. If one solves the equation for "X" (the ratio of oxygen radical consumption by the scavenger), the formula is reduced to :

$X = (I_o - I)/I_o$.

The formula for "X" can then be inserted into formula (8) which is the concentration of the scavenger "[S]". When this formula is solved, the result is:

$I_o / I = ([S] + ID_{50})/ ID_{50}$.

Based on this formula and knowing that the "ID_{50}" of citrulline is 0.00434 M as set forth in Table 1, as well as the reaction rate consistent that is also provided in Table 1, a concentrations of 0.5 mM citrulline can provide a value of 89.7 percent of the control and a concentration of 1 mM citrulline can provided a value of 81.3 percent of the control. Thus, based on the information provided in the specification and Figure 4, applicants respectfully submit that the claims of record are fully supported by the present application.

The claims now of record are neither disclosed nor suggested by the prior art set forth in the Official Action. In particular, JP 9-241637 which has been referred to in the Official Action as "Okubo", relates to a composition capable of removing active oxygen free radicals using a composition that comprises an active oxygen free radical scavenger and one or more substances selected from uronic acids, uronides, mucopolysaccharides and amino acid-peptides. While citrulline is included amongst

[1] Bulletin Chemical Society of Japan, 63, 187-191 (1990).

the amino acids set forth in paragraph [0071], it is not disclosed as being the active oxygen preradical scavenger, but rather as an auxiliary agent. This understanding is made clear from this same paragraph which indicates that if the amino acid is combined with an active oxygen radical scavenger, it will demonstrate a strong effect. However, Okubo itself teaches that citrulline by itself does not have a strong scavenger effect. This is made apparent from the Table in paragraph [0096] which shows that for citrulline by itself (citrulline is the second to last material under component "Z"), the effect is "VW" at all the tested pH values which means that the effect is very weak. It is only when the materials are combined with the separate oxygen scavenger "EGC" (epigallocatechin) that acceptable results are obtained.[2]

With a clear understanding of what Okubo fairly discloses, those of ordinary skill in the art will recognize that the claims of record are patentably distinct. Okubo requires the presence of an oxygen scavenger and uses auxiliary compounds, one of which can be citrulline. Thus, the document clearly does not meet the presently claimed methods which require that the composition or method consists essentially of citrulline in the defined amount. Therefore follows that the claims are patentable over the fair teachings of Okubo.

Published International Application No. WO 95/15147 which the Examiner has referred to as "Ennen", describes the use of L-arginine, L-ornithine or L-citrulline or their salts, acid addition salts, esters or amides, as needed with the addition of folic acid or its salts and/or one or more compounds selected from the group of flavins, for prophylaxis and/or treatment of neurosensory phenomena. In relying on this

[2] See paragraphs [0040]-[0042] for the description of epigallocatechin and other similar compounds as the oxygen radical scavenger.

document, the Examiner has referred to page 16, formulation 6 which includes 143 mM of citrulline in the composition. The Examiner has conceded that Ennen does not expressly mention a method of treating skin by applying a citrulline-containing composition as recited in claim 19, but has asserted that such use is inherent.

As recognized by the Examiner, Ennen does not teach the scavenging of oxygen by the use of citrulline and clearly does not teach such use by the defined amount of the material. Indeed, the formulation relied on on page 16 of Ennen is not designed to scavenge oxygen and contains substantially more citrulline than the amount set forth in the claims of record which have been shown to be effective by the evidence provided in the specification, such as in Figure 4. Thus, Ennen also cannot be used to reject any of the claims now of record.

Since claim 21, as well as claims 22-24, have been canceled without prejudice or disclaimer, the rejection under 35 U.S.C. §103(a) of claim 21 has been rendered moot. However, in view of the substantial differences between the respective documents and the claims now of record, applicants respectfully maintain that the claims are patentable over the combination of prior art used to reject claim 21, even assuming that there is a proper basis for combining the respective teachings of the documents.

With respect to the rejection of claims 26 and 27 under 35 U.S.C. §103(a) based on U.S. Patent No. 5,874,471 which has been referred to as "Waugh", this patent describes the orthomolecular medical use of L-citrulline for vasoprotection, relaxative smooth muscle tone and cell protection. As set forth in the Abstract, the method provides administration of "sizable amounts" of L-citrulline as a precursor

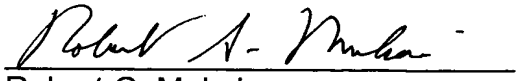
substance for bioconversion to L-arginine. This method does not constitute a method to improve preservation of an oxygen phobic substance as recited in claim 26. Moreover, the disclosed method is specifically designed for oral ingestion to convert the L-citrulline to L-arginine and therefore does not relate to a method of decreasing the active oxygen contact of an active oxygen phobic substance as specifically recited in the claim 26 by adding citrulline in the defined amount. In this regard, claim 27, which defines specific active oxygen phobic substances that are treated to decrease the active oxygen content thereof, is even further removed from Waugh. Thus, claim 26 and the claims dependent there from are also patentable over Waugh.

For all of the reasons set forth above, applicants respectfully submit that the claims of record are patentable over the cited prior art and therefore request reconsideration and allowance of the present application.

Should the Examiner wish to discuss any aspect of the present application, she is invited to contact the undersigned attorney at the number provided below.

Respectfully submitted,

BUCHANAN INGERSOLL & ROONEY PC

By: 

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The Application of ESR Spin-Trapping Technique to the Evaluation of SOD-*like* Activity of Biological Substances

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The SOD-*like* activity of several biological substances was evaluated by an ESR spin-trapping technique. Superoxide radicals (O_2^-) were supplied enzymatically from a hypoxanthine–xanthine oxidase reaction to the evaluating system. By using a spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), the generated O_2^- was trapped stoichiometrically (1:1) as the spin adduct of O_2^- (DMPO–O_2^-). When biological substances were added to the system, a decrease in the ESR signal intensities of the adducts was observed. This phenomenon could be explained as being an inhibition of adduct formation, and related to the reactivity of added biological substances with O_2^-, called an SOD-*like* activity. By the method of kinetic competition with a 50% inhibitory dose (ID_{50}), the second-order rate constant for the reaction between O_2^- and biological substance was determined. These rate constants can be used as a measure of the reactivity.

Superoxide radicals (O_2^-) are generated from molecular oxygen or hydrogen peroxide by an one-electron transfer reaction.[1] This radical induces various injuries to the surrounding organism.[2,3] Therefore, removing O_2^- is probably one of the most effective defences of a living body against oxidative stress.[2,3] In 1969, McCord and Fridovich found superoxide dismutase (SOD) to be a scavenger of O_2^-.[4]

Superoxide radical generations and SOD activities in biological systems are commonly measured by optical spectrometry using cytochrome *c*, tetranitromethane, epinephrine, nitroblue tetrazolium (NBT), pyrogallol, NADH + lactate dehydrogenase (LDH), ascorbate, hydroxylamine, and 6-hydroxydopamine.[4–10] However, there are some questions about these methods: the low selectivity against O_2^- and/or an obstruction by the coexistence of insoluble particles and colored impurities, such as chromoproteins.[2,3]

Superoxide radicals are also detected at low temperatures by ESR spectrometry using a rapid-freezing technique or at room temperature by a spin-trapping technique. Especially, the spin-trapping technique[11] is useful for discriminating trapped radical species, and various short-lived radical intermediates are identified by this technique.[12] In the process of developing this technique, many workers have reported reactions between specific radical species and their scavengers.[13–17] Recently, part of these studies was applied to an assay of SOD activity.[18,19] This SOD assay method can be used to analyze crude samples without any purification, since the color and turbidity do not affect the measurements.[19]

There have been some reports that coexisting substances, such as L-ascorbic acid or celuroplasmin, hinder accurate measurements of SOD activity.[2] This means that they have an SOD-*like* activity in playing important roles, namely, biological defences against O_2^-.[8,20] On the other hand, it has been reported that in competition reactions for O_2^- between 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) and SOD, the formation of the adduct (DMPO–O_2^-) was suppressed by SOD, and the second-order rate constant of DMPO, itself, was estimated from the inhibitory effect of SOD.[13,14] Here, we expand this method to evaluate the SOD-*like* activity of various biological substances.

Experimental

Materials. A nitrone spin trap [5,5-dimethyl-1-pyrroline *N*-oxide (DMPO, Mitsui Toatsu Chemicals)], a chelator for trace metal impurities [diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DETAPAC, Wako Pure Chemical)], a superoxide radical source [hypoxanthine (HPX, Sigma Chemical) and xanthine oxidase (XOD, Boehringer Mannheim, cow milk)], and a primary standard of spin concentration [4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL, Sigma Chemical)] were used.

Radical scavengers [copper-zinc superoxide dismutase (Cu,Zn–SOD, Boehringer Mannheim, bovine erythrocyte), manganese superoxide dismutase (Mn–SOD, Sigma Chemical, *Escherichia coli*), iron superoxide dismutase (Fe–SOD, Sigma Chemical, *Escherichia coli*), ceruloplasmin (Alpha Therapeutic, human serum), ascorbate oxidase (Toyobo, type III, cucumber), ferricytochrome *c* (Sigma Chemical, type VI, horse heart), peroxidase (Toyobo, type I-C, horseradish), catalase (Boehringer Mannheim, type I, beef liver), and L-ascorbic acid (Daiichi Pure Chemical)] were also used. The other chemicals used were of the highest grade commercially available.

Instruments. ESR spectra were recorded on a JEOL JES-RE1X spectrometer using an aqueous quartz flat cell (a JEOL LC-12 ESR cuvette, inner size 60 mm×10 mm×0.31 mm, effective volume 160 μl). Optical absorption spectra were measured by an Otsuka Electronics MCPD-100 multi-channel photodetector.

Preparation of Samples. Superoxide radicals were generated from a hypoxanthine–xanthine oxidase (HPX–XOD) reaction system.

The sampling procedure was as follows: One hundred mM (1 M=1 mol dm^{-3}) sodium phosphate buffer solution (pH 7.8) was used for a solvent. A solution of 2.0 mM HPX (a),

5.5 mM DETAPAC (b), various concentrations of biological substance (c) and 0.33 unit/ml XOD (d) was prepared before use. The XOD solution was stored in an ice bath so as to prevent any inactivation of enzyme.

Fifty μl of a, 35 μl of b, 50 μl of c (or solvent) and 15 μl of DMPO were put into a test tube. To the mixed solution, 50 μl of d was added. After quick stirring, 200 μl of the mixture was taken into a flat cell. The mixture contained 0.50 mM HPX, 0.96 mM DETAPAC, 0.67 M DMPO,[21] 0.083 unit/ml XOD and an adequate concentration of the biological substance.

Measurements. The enzyme activity of Cu,Zn-, Mn-, and Fe-SOD was calibrated by the method of McCord and Fridovich.[6] The superoxide radical generation in our system was confirmed by the reduction of ferricytochrome *c* using the absorbance change at 550 nm.[22]

A quantitative analysis of DMPO-O_2^- by ESR spectrometry was performed under the following conditions for obtaining a high reproducibility of the spin adduct yields: Recording of the ESR spectrum started 40 s after the addition of XOD. The recording rate was 5 mT·min^{-1}. After recording, the signal intensity of the lowest field peak of the spectrum (about 85 s after the addition of XOD) was normalized as a relative height against the standard signal intensity of the manganese oxide marker (MnO). An absolute concentration of DMPO-O_2^- was finally determined by a double-integration of the ESR spectrum. One μM TEMPOL solution was used for a primary standard of ESR absorption.

Results and Discussion

Reactivity of Several Biological Substances with O_2^-. When DMPO was added to a solution of the HPX-XOD reaction system, the spin adduct, DMPO-O_2^-, was formed.[13] Figure 1(a) shows a typical ESR spectrum of DMPO-O_2^- obtained under controlled conditions. Hyperfine coupling constants of the signal were analyzed as one nitrogen, a_N=1.41 mT, one hydrogen of β-position, $a_{H\beta}$=1.14 mT, and one hydrogen of γ-position, $a_{H\gamma}$=0.13 mT.[13] At the same time, a small amount of the hydroxyl radical adduct (DMPO-OH, $a_N=a_{H\beta}$=1.48 mT) was observed.[13,16] When Cu,Zn-SOD of various concentrations was added to the system, the signal intensities of DMPO-O_2^- decreased with an increase in the SOD concentration, as shown in Fig. 1(b) to (e). This phenomenon suggests that the reaction between O_2^- and DMPO is inhibited by Cu,Zn-SOD. According to Finkelstein et

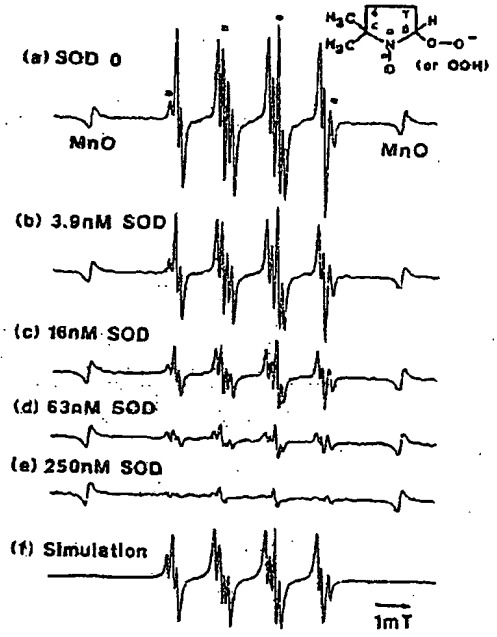

Fig. 1. ESR spectra of DMPO-O_2^- formed from the HPX-XOD system. The medium contained 0.50 mM HPX, 0.96 mM DETAPAC, 0.67 M DMPO, 0.083 unit/ml XOD, various concentrations of Cu, Zn-SOD and 100 mM sodium phosphate at pH 7.8, 23°C. A small amount of DMPO-OH (*) was found in the spectra. Modulation amplitude was 0.05 mT (100 kHz), recording range 10 mT, recording time 2 min, time constant 0.1 s, microwave power 8 mW (9.414 GHz). Simulation spectrum consisted of 90% DMPO-O_2^- and 10% DMPO-OH.

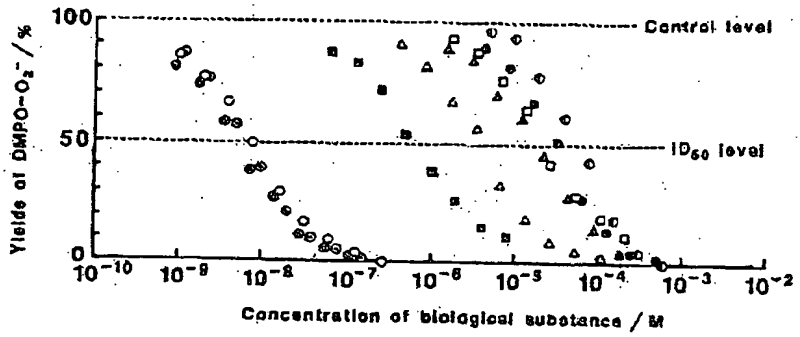

Fig. 2. Inhibitory effect of various biological substances on the formation of DMPO-O_2^-: Cu,Zn-SOD (○), Mn-SOD (⊙), Fe-SOD (◐), ceruloplasmin (□), ascorbate oxidase (△), ferricytochrome *c* (●), peroxidase (■), catalase (▲), and L-ascorbic acid (◐).

al.,[13,14] this phenomenon is a competition reaction between DMPO and SOD for O_2^-.

A phenomenon similar to the addition of Cu,Zn-SOD occurred widely in the addition of various biological substances such as Mn-SOD, Fe-SOD, ceruloplasmin, ascorbate oxidase, ferricytochrome *c*, peroxidase, catalase, and L-ascorbic acid. Figure 2 shows the relationship between the signal intensities of the DMPO–O_2^- observed and the concentrations of the biological substances added. Among the biological substances, the patterns of inhibitory effects were similar, but the effective concentration ranges were different.

The inhibitory effect of the biological substances was in the order: SOD > peroxidase > ascorbate oxidase > catalase ≧ ceruloplasmin ≧ ferricytochrome *c* ≧ L-ascorbic acid.

Stoichiometry between Generated O_2^- and Formed Spin Adduct. When DMPO of various concentrations was added to the HPX-XOD system, the signal intensities of DMPO–O_2^- increased with an increase in the DMPO concentration when it was less than 0.5 M and were independent of the DMPO concentration when it was more than 0.5 M. Figure 3 shows the relationship between the concentrations of DMPO–O_2^- formed and DMPO added. The figure indicates that the DMPO concentration shown in Fig. 1(a) (0.67 M, the control condition, see Experimental) was at a saturating level for generated O_2^-. This observation indicates that almost all O_2^- is trapped by DMPO under the conditions specified in Fig. 1(a).[13,14]

This result was compared with the reduction rate of ferricytochrome *c*. Figure 4 shows the reduction rate of ferricytochrome *c* (▲) when 0.53 mM ferricytochrome *c* was added to the HPX-XOD system instead of 0.67 M DMPO. The concentration of DMPO–O_2^- (●) measured under the conditions specified in Fig. 1(a) was overlapped on the plots. In the experiment, the concentration of ferricytochrome *c* was sufficient for scavenging O_2^-, because 0.53 mM ferricytochrome *c* can almost suppress adduct formation, as shown in Fig. 2. This fact means that the reduction rate of ferricytochrome *c* by O_2^- is nearly equal to the generation rate of O_2^- of the system.[22] In the figure, the concentrations of reduced cytochrome *c* (ferrocytochrome *c*) and formed DMPO–O_2^- increased to 18 μM after about 90 s, and the time course of DMPO–O_2^- within the period also agreed with that of ferrocytochrome *c*.

Thus, the correlation between the concentrations of formed ferrocytochrome *c* and formed DMPO–O_2^- clearly showed that: (1) the O_2^- generated from the HPX-XOD system changed rapidly and stoichiometrically (1:1) into the DMPO–O_2^- under this experimental condition, and (2) a kinetic study of generated O_2^- using the signal intensities of DMPO–O_2^- is possible at an early stage of the reaction.

Evaluation of Second-Order Rate Constant for the Reaction between O_2^- and Biological Substance. Based on the obtained results, we modified the kinetic competition models[8,13,14,19,22] for our experiments. It is useful to consider the first step of the scavenging reaction of O_2^- as a contact reaction between O_2^- and its reactant. Assuming that the contact reaction of O_2^- to DMPO or scavenger (S) is of second-order, the first step of each reaction can be described as

$$O_2^- + \text{DMPO} \xrightarrow{k_1} \text{DMPO–}O_2^- \qquad (1)$$

and

$$O_2^- + S \xrightarrow{k_2} S\text{–}O_2^-. \qquad (2)$$

where k_1 and k_2 are second-order rate constants for

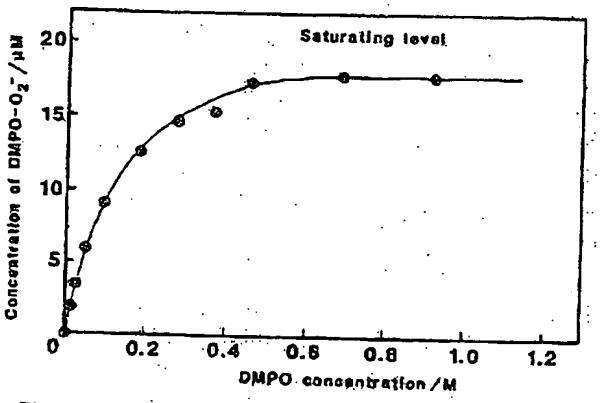

Fig. 3. Yields of DMPO–O_2^- as a function of DMPO concentration in the HPX-XOD reaction system.

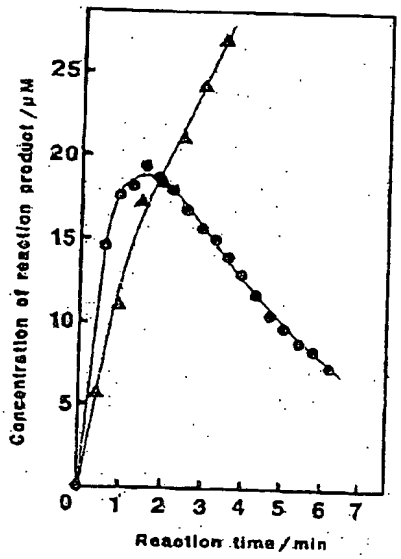

Fig. 4. Time course of product formation in the HPX-XOD reaction system: Ferrocytochrome *c* (▲) and DMPO–O_2^- (●).

reactions 1 and 2, respectively. Then, the initial rate of each reaction is given by

$$\frac{d[DMPO\text{–}O_2^-]}{dt} = k_1 \cdot [DMPO] \cdot [O_2^-] \quad (3)$$

and

$$\frac{d[S\text{–}O_2^-]}{dt} = k_2 \cdot [S] \cdot [O_2^-] \quad (4)$$

A spontaneous disproportionation of O_2^- often causes a scavenger-independent decrease of DMPO–O_2^-. In our experiments, however, such a decrease was insignificant. The superoxide-scavenging effect caused by an added scavenger has its main contribution to the intensity change of DMPO–O_2^-, since the DMPO concentration (0.67 M) is sufficiently high to trap almost all of the O_2^- under our experimental conditions (as discussed in the preceding section). A similar consideration was first reported by Sawada and Yamazaki.[20] Thus, when reactions 1 and 2 compete with each other, the reaction rate in the initial stage can be expressed simply by

$$\frac{d[DMPO\text{–}O_2^-]}{dt} : \frac{d[S\text{–}O_2^-]}{dt} = (1-X) : X, \quad (5)$$

where variable X ($0<X<1$) is the ratio of O_2^- consumption by the added scavenger. From Eqs. 3, 4, and 5, the following equation can be derived:

$$k_2 = k_1 \cdot \frac{X}{(1-X)} \cdot \frac{[DMPO]}{[S]}. \quad (6)$$

In the case of 50% inhibition ($X=0.5$), a conventional measure of the inhibition, ID_{50} (inhibitory dose-fifty), can be used.[4–6,9,10,15,20] Equation 6 can be simplified as

$$k_2 = k_1 \cdot \frac{[DMPO]}{ID_{50}}. \quad (7)$$

In the above equation, [DMPO] and ID_{50} are the experimental values, and k_1 at pH 7.8 is obtained from Ref. 14 to be 18 $M^{-1}\,s^{-1}$. Therefore, by using [DMPO], ID_{50} and k_1 as known values, we can estimate an approximate value of unknown k_2.

On the other hand, ID_{50} can be related to [S] by using variable X (see Eqs. 6 and 7) as

$$[S] = \frac{X}{(1-X)} \cdot ID_{50}, \quad (8)$$

where $X/(1-X)$ means the ratio of the competition between scavenger (S) and the spin trap (DMPO). In addition, from the definition of variable X, the spin concentration of DMPO–O_2^- in the early stage of the reaction is written by

$$I = (1-X) \cdot I_0, \quad (9)$$

where I and I_0 are the spin concentrations of DMPO–O_2^- in the presence and absence of a scavenger, respectively. The I_0 is also a constant peculiar to the superoxide generating system.

Based on Eqs. 8 and 9, the inhibitory effect of biological substances can be simulated by computer. When simulations were carried out, I and [S] were

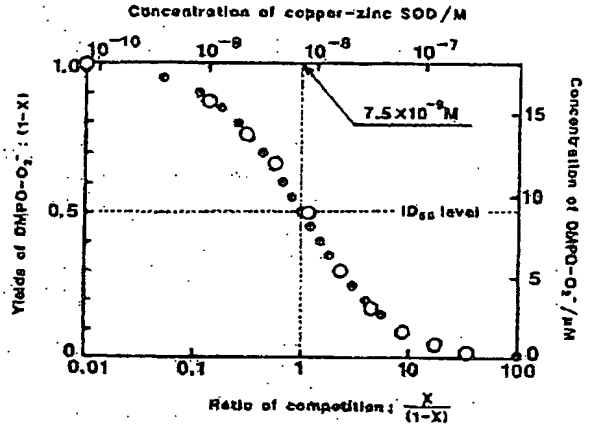

Fig. 5. Inhibitory effect of Cu,Zn-SOD on the formation of DMPO–O_2^-: Experimental (○) and simulated (●) values, where I_0 and ID_{50} used were 1.8×10^{-6} M and 7.5×10^{-9} M, respectively.

Table 1. ID_{50} and Second-Order Rate Constants of Various Biological Substances

Substance	ID_{50}	ID_{50}[b]	k_2	k_{rel}	pH_{rel}	Ref.
	M	mg ml^{-1}	$M^{-1}\,s^{-1}$	$M^{-1}\,s^{-1}$		
Cu,Zn-SOD[a]	7.5×10^{-9}	0.00024	1.6×10^{9}	ca. 2×10^{9}	5—9.5	25,26
Mn-SOD[a]	6.5×10^{-8}	0.00026	1.9×10^{9}	1.8×10^{9}	7.8	24
Fe-SOD[a]	5.1×10^{-9}	0.00020	2.4×10^{9}	1.6×10^{9}	7.8	24
Ceruloplasmin[a]	1.5×10^{-8}	2.0	8.1×10^{8}	3.04×10^{8}	7.8	27
Ascorbate oxidase[a]	2.5×10^{-6}	0.35	4.8×10^{6}			This work
Ferricytochrome c[a]	2.7×10^{-6}	0.33	4.5×10^{6}	6.2×10^{6}[d]	7.8	28
Peroxidase[a]	5.0×10^{-7}	0.020	2.4×10^{7}	1.6×10^{6}[d]	7—8.8	29
Catalase[a]	1.3×10^{-5}	3.0	9.6×10^{5}	ca. 2×10^{6}	7.4	30
L-Ascorbic acid	3.4×10^{-5}	0.0060	3.5×10^{5}	2.7×10^{5}	7.4	8

a) The following molecular weights were used: Cu,Zn-SOD (32000), Mn-SOD (40000), Fe-SOD (39000), ceruloplasmin (134000), ascorbate oxidase (140000), ferricytochrome c (12400), peroxidase (40000), and catalase (240000). b) The molarity of ID_{50} was calculated from this value. c) Calculated from the data of Ref. 28. d) Rate constant for the reaction between horseradish peroxidase Compound I and O_2^-.

replaced by $(1-X)\cdot I_0$ and $\{X/(1-X)\}\cdot ID_{50}$, respectively. The I_0 and ID_{50} were fixed at suitable values which led to a best fit to the experimental data. Simulation values were calculated by changing variable X from 0.01 to 0.99 at intervals of 0.05. Figure 5 shows both simulated (●) and experimental (○) values of Cu,Zn–SOD. Both values agreed well and the validity of our treatment was confirmed. By the simulation, the ID_{50}'s of Cu,Zn–SOD could be evaluated more exactly. The ID_{50}'s of the other substances were determined in the same manner.

Table 1 shows the ID_{50}'s, evaluated rate constants (k_2) and reported rate constants (k_{ref}) of several biological substances, where k_{ref} of Refs. 25—30 were the values obtained directly by a pulse radiolysis method; the others were indirectly obtained by the kinetic competition method. In the table, the rate constants of Cu,Zn–SOD, Mn–SOD, Fe–SOD, ceruloplasmin, ferricytochrome *c*, peroxidase, catalase and L-ascorbic acid agree with the reported values within one order of magnitude.[31]

Thus, we concluded that: (1) the original k_1 in Ref. 14 was really an exact value; (2) our method gave reasonable values as k_2; (3) the concentration of DMPO essentially did not affect the reactivity of above biological substances; (4) there was no reference of the reactivity between ascorbate oxidase and O_2^-, but the ascorbate oxidase showed a strong reactivity with O_2^-, and (5) these rate constants became a good measure of the reactivity.

In general, the ESR spin-trapping technique could scarcely be used for a kinetic investigation of the reactivity between an activated short-lived radical intermediate and its scavenger. This new kinetic analysis technique will be applied to an investigation of the SOD-*like* function of drugs.

We are grateful to Dr. Ikuko Ueno of the University of Tokyo, Institute of Medical Science for her helpful suggestion to our biochemical experiments. We also wish to express our thanks to Dr. Kiyoko Yamamoto of the Institute of Physical and Chemical Research (RIKEN) for her valuable advice on arranging this work.

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